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Protective immunization of hamsters against *Opisthorchis viverrini* infection is associated with the reduction of TGF-β expression

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ABSTRACT

Opisthorchis viverrini infection is a significant health problem in Thailand and other countries in Southeast Asia. There is little known about the mechanisms of the immune response to *O. viverrini* in immunoprotection. However, it has been reported that this parasite can suppress both cell and antibody mediated immune responses. The TGF-β and IL-10 are immunosuppressive cytokines that play an important role in inhibition of host immune response leading to worm survival. In this study, we immunized hamsters to protect against *O. viverrini* infection and the IL-4, IL-10, TGF-β and IFN-γ expression in spleen was investigated by real time PCR analysis. An *O. viverrini*-crude somatic antigen preparation (CSAg) administered with complete Freund's adjuvant (CFA) or with alum was used to stimulate immune responses in *O. viverrini*-primed hamsters. The greatest percent protection (48.4%) was seen following immunization with CSAg plus alum. The mean number ± SD of worms recovered in the PBS control, CFA alone, CSAg plus CFA, alum alone and CSAg plus alum was 17.4 ± 2.3, 17.1 ± 3.3, 14.5 ± 3.8, 14.5 ± 2.3 and 9 ± 2.7, respectively. Significant protection correlated with the reduction of TGF-β and IL-10, but not IL-4, IFN-γ expressions. Since TGF-β expression is significantly increased in the spleens of hamsters with opisthorchiasis, stimulation of this cytokine by parasite antigens was confirmed by using CSAg and primary hamster spleen cells. Antigen fractions with molecular masses of 81–92, 64–72 and 19–21.4 kDa were found to significantly induce TGF-β production. Our results suggested that TGF-β induction by *O. viverrini* may have an important role in parasite survival.

1. Introduction

Opisthorchiasis, caused by the liver fluke *Opisthorchis viverrini*, is endemic to many countries around the Mekong River including Thailand, Laos, and Cambodia (Giboda et al., 1991; Harinasuta et al., 1993). For example, in Thailand, the prevalence of opisthorchiasis in 2001 was 19.3 and 15.7% in the North and Northeastern regions, respectively (Jongsuksuntigul and Imsomboon, 2003). *O. viverrini* can cause hepatobiliary disease and induce a high antibody titer and strong cell mediated immune responses. Moreover, it has been shown that opisthorchiasis is an important risk factor for cholangiocarcinoma (Thamavit et al., 1994; Vatanasapt et al., 1990).

To date, the hamster appears to be the best model for studying *O. viverrini* infection (Bhamarapravati et al., 1978), however, resistance to *O. viverrini* challenge in the hamsters can be induced only in animals harboring a low number of worms (Flavell, 1982). *O. viverrini* can suppress both cell and antibody mediated immune responses (Wongratanacheewin et al., 1987). Suppression is most severe in prolonged infections, but it can be abolished by anthelmintic treatment (Wongratanacheewin et al., 1987). This finding suggests that the immunosuppressive effect is reversible and is associated with active infection. We previously reported that the parasite stimulates the expression of the Th1-inducing cytokine, IL-12, in the early stage of infection (2 weeks post infection) and the expression of the Th2-inducing cytokine, IL-4, and the regulatory cytokines, TGF-β and IL-10, are significantly increased in chronic and/or heavy infections (Jittimanee et al., 2007). It has been demonstrated that TGF-β and IL-10 are immunosuppressive cytokines that play an important role in inhibition of host immune response leading to worm survival (Maizels and Yazdanbakhsh, 2003). We therefore hypothesized that the *O. viverrini* antigens stimulate TGF-β and IL-10 cytokine production which down-regulates the host immune response leading to worm survival. To test this hypothesis, we used a modified immunization

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protocol designed to obtain the optimal protection in hamsters and assessed cytokine expression. Since *O. viverrini* does not normally invade tissues during development in mammalian hosts, the chance of stimulating the host immune system is therefore minimal. None of the attempts to induce acquired immunity against *O. viverrini* in uninfected hamsters have been successful (Sirisinha et al., 1983). However, the host that primed with low dose (5 metacercaria) before immunization could provide some degree of protection (Sirisinha, 1984). We thus used low dose pre-exposure to prime the mucosal immune system before immunization as a model to study profile of protective immunity. Two adjuvants, Complete Freund's adjuvant (CFA), thought to be a Th1 inducer (Yip et al., 1999), and alum, reported as a Th2 cytokine inducer (Pollock et al., 2003) were used in combination with parasite antigen to induce protective immunity.

2. Materials and methods

2.1. Research design

Five groups of 10 male golden Syrian hamsters were used for this study. They were housed in the animal care unit at Faculty of Medicine, Khon Kaen University on a 12 h light/dark cycle and fed a commercial feed *ad libitum*. Animals from groups 1–5 were infected orally with 5 metacercaria (Mc) 3 months prior to immunization as described previously (Sirisinha and Wongratanacheewin, 1986). Group 1 was then injected intraperitoneally with phosphate buffer saline (PBS). Groups 2–5 were injected intraperitoneally with CFA plus PBS, CFA mixed with 200 μg crude somatic antigens (CSAg), alum plus PBS, or alum mixed with 200 μg CSAg, respectively (Table 2). Two weeks after injection, all hamsters were challenged orally with 25 Mc. Animals were killed 3 months post-challenge at which time the number of adult worms and eggs per gram feces were counted and spleen cells and sera were collected for cytokine determination. Cytokine (TGF-β, IFN-γ, IL-4, and IL-10) expression was measured in the group that had the best protection (Group 5) by real time PCR and ELISA (for TGF-β). Animal use, care, and handling were done in accordance with the National Research Council of Thailand Guidelines for the care and use of animals for scientific purposes. All animal experimental protocols were approved by Faculty of Medicine Animal Ethics Committee, Khon Kaen University, Khon Kaen, Thailand.

2.2. Worm recovery

Adult parasites were recovered from the bile duct of infected hamsters and the number of worms was determined. The percent worm reduction was calculated using the equation:

$$\text{Worm reduction}\,(\%) = \frac{W_n - W_i}{W_n} 100$$

W_n is the mean number of worm recovery in PBS immunized group (Group 1) and W_i is the number of worms recovered in other immunized groups.

2.3. Cytokine mRNA positive control

RNAs used for preparation of the positive controls and for generation of cRNA standard curves in real-time PCR assays were prepared as described previously (Jittimanee et al., 2007). Briefly, isolated spleen cells from normal uninfected hamsters were cultured at a concentration of 1×10^6 cells/ml and stimulated with 10 μg/ml of Concanavalin A (Con A) (Sigma–Aldrich, St. Louis, MO) in RPMI 1640 medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml). After 24 h of stimulation, the spleen cells were harvested and total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA).

2.4. Preparation of RNA for cytokine assays

Spleens were removed from each hamster and immediately frozen on dry ice. RNA was extracted using Trizol reagent according to the manufacturer's recommendations. In brief, spleen tissue was homogenized in Trizol reagent and incubated for 5 min at 30 °C, centrifuged at 10,000 × *g* for 10 min at 4 °C, and the supernatant was removed to a new tube. Chloroform–Trizol mixture (0.2:1) was added and the homogenates were shaken vigorously for 15 s before incubatation at 30 °C for 3 min. The homogenates were then centrifuged at 10,000 × *g* for 15 min at 4 °C. The RNA in the aqueous phase was precipitated by the addition of isopropyl alcohol. After centrifugation, the RNA pellet was washed with 75% ethanol. Contaminating DNA was digested with RNase-free DNases (Promega, Madison, WI) at 37 °C for 30 min. The RNA quality was evaluated by agarose gel electrophoresis and by determining the optical density 260/280 nm ratio; the concentration was estimated by measuring the optical density at 260 nm.

2.5. Primers for cytokine determination

The primers for PCR amplification of IFN-γ, IL-4, IL-10, TGF-β and hypoxanthine phosphoribosyl transferase (HPRT) (used for either cRNA construction or real time PCR) have been described previously (Jittimanee et al., 2007). The sequences, accession numbers, primers and amplification conditions are shown in Table 1.

2.6. cDNA and cRNA construction for standard real-time PCR

The cDNA construction was carried out by converting the total RNA to single-stranded cDNA using M-MLV reverse transcriptase (Invitrogen, CA) (Fronhoffs et al., 2002). T7 promoter sequences (TAATACGACTCACTATAGGGA) and oligo dT (Table 1) were then added to the 5′ end of the forward primer to the 3′ end of the reverse primer respectively by PCR amplification to generate cRNA. The 25 μL PCR reactions contained 50–100 ng of cDNA, 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8.3), 1 unit of *Taq* DNA polymerase (Invitrogen Life Technologies, CA), 0.2 mM each dNTP, and 1.5 mM $MgCl_2$. The PCR amplification conditions are shown in Table 1. The PCR products containing the T7 promoter were used as templates for *in vitro* transcription using a MEGAscript® kit (Ambion Inc., Austin, TX). DNase I (2 Unit) (Ambion Inc.) was added to remove the template DNA. The concentration of the cRNA generated was determined spectrophotometrically at 260 nm and the copy number of the cRNAs was calculated as described previously (Fronhoffs et al., 2002).

2.7. Real-time PCR

Ten-fold serial dilutions of a standard cRNA (obtained from Con A stimulated hamster spleen cells) were made to obtain 10^1 to 10^8 template copies per reaction before reverse transcription to cDNA using M-MLV reverse transcriptase (Invitrogen, USA). The complementary DNA was generated to each dilution of cRNA (or 2 μg of total RNA) according to the manufacturer's recommendations. All cDNAs were kept at −20 °C prior to real-time PCR using a Light-Cycler® machine (Roche Applied Science, Indianapolis, IN). The real-time amplification conditions were the same as described previously (Jittimanee et al., 2007). The results were expressed as the of copy number of cytokine gene to HPRT ratio.